

Appl. No.: 09/837,235

Docket No. 289550-122 US2

Amdt. dated May 28, 2004

Reply to Final Office Action dated Mar. 31, 2004

Page 26 of 26

APPENDIX A

Attached is a Replacement Sheet for Figures 15A-B and 16 A-D.

21/26

C. Antarctica Lipase B Nucleotide and Amino Acid Sequence

```

      10      20      30      40      50      60
ctaccttcgggttcggaccctgccttttgcagcccaagtcgggtgctcgatgcgggtctg
  L  P  S  G  S  D  P  A  F  S  Q  P  K  S  V  L  D  A  G  L
      70      80      90     100     110     120
acctgccagggtgcttcgccatcctcgggtctccaaacccatccttctcgtcccggaacc
  T  C  Q  G  A  S  P  S  S  V  S  K  P  I  L  L  V  P  G  T
     130     140     150     160     170     180
ggcaccacaggtccacagtcgttcgaotcgaactggatccccctctcaacgcagttgggt
  G  T  T  G  P  Q  S  F  D  S  N  W  I  P  L  S  T  Q  L  G
     190     200     210     220     230     240
tacacaccctgctggatctcaccctcccggttcattgctcaacgcacccagggtcaacag
  Y  T  P  C  W  I  S  P  P  P  F  M  L  N  D  T  Q  V  N  T
     250     260     270     280     290     300
gagtacatggtcaacgccatcaccgcgctctacgctgggttcgggcaacaacaagctccc
  E  Y  M  V  N  A  I  T  A  L  Y  A  G  S  G  N  N  K  L  P
     310     320     330     340     350     360
gtgcttaacctggtcccagggtgggtctggttgacagtggggtctgaccttcttcccagt
  V  L  T  W  S  Q  G  G  L  V  A  Q  W  G  L  T  F  F  P  S
     370     380     390     400     410     420
atcagggtccaaggctcgatcgacttatggcctttgcgcccgcactacaaggggcaccgtctc
  I  R  S  K  V  D  R  L  M  A  F  A  P  D  Y  K  G  T  V  L
     430     440     450     460     470     480
gccggccctctcgatgcactcgcgggttagtgacccctccgtatggcagcaaacaccagggt
  A  G  P  L  D  A  L  A  V  S  A  P  S  V  W  Q  Q  T  T  G
     490     500     510     520     530     540
tcggcactcaccaccgcactccgaaacgcaggtgggtctgacccagatcgtgccaccacc
  S  A  L  T  T  A  L  R  N  A  G  G  L  T  Q  I  V  P  T  T
     550     560     570     580     590     600
aaactctactcggcgaccgcagagatcgttcagcctcagggtgtccaactcgccactcgac
  N  L  Y  S  A  T  D  E  I  V  Q  P  Q  V  S  N  S  P  L  D
     610     620     630     640     650     660
tcactctacctcttcaacggaaagaacgtccaggccacaggccggtgtgtgggcggtgttc
  S  S  Y  L  F  N  G  K  N  V  Q  A  Q  A  V  C  G  P  L  F
     670     680     690     700     710     720
gtcatcgaccatgcaggtcgcctcaccctcgcagttctctctacgtcgtcggtcgatccgcc
  V  I  D  H  A  G  S  L  T  S  Q  F  S  Y  V  V  G  R  S  A
     730     740     750     760     770     780
ctgcgctccaccacgggcccaggctcgttagtgacagactatggcattacggactgcaaccct
  L  R  S  T  T  G  Q  A  R  S  A  D  Y  G  I  T  D  C  N  P
     790     800     810     820     830     840
cttcccgccaatgatctgactcccgagcaaaagggtcgccgcggtcgcgctcctggcgccg
  L  P  A  N  D  L  T  P  R  Q  K  V  A  A  A  A  L  L  A  P
     850     860     870     880     890     900
gcagctgcagccatcgtggcggtccaaagcagaactgcgagcccgacctcatgccttac
  A  A  A  A  I  V  A  G  P  K  Q  N  C  R  P  D  L  M  P  Y
     910     920     930     940     950
gccgcgccctttgcagtaggcaaaaggacctgctccggcatcgtcacccctga(SEQ ID NO: 1)
  A  R  P  F  A  V  G  K  R  T  C  S  G  I  V  T  P  * (SEQ ID NO: 2)

```

FIG. 15A

22/26

PCR Oligos for *Candida antarctica* Lipase B**Oligos for pPal-CALB**

Primer A: 5'atg gga att cca tca tca tca tca tca cag cag egg cct acc ttc egg ttc gga ccc3' (SEQ ID NO: 3)

Primer B: 5'cta ttg gcg goa gcc tat cag ggg gtc aag atg cag gc3' (SEQ ID NO: 4)

Oligos for Point Mutations (made in pPal-CALB)**M1- F9Y**

Primer M1P: 5'atg gga att cca tca tca tca tca tca cag cag egg cct acc ttc egg ttc gga ccc3' (SEQ ID NO: 5)

M2- W52YPrimer M2P: 5'cga ctc gaa cta Caa ccc cct ctc3'
(SEQ ID NO: 6)

Primer M2R: 5'gag agg ggg atG Tag ttc gag tgg3' (SEQ ID NO: 7)

M3- F117YPrimer M3P: 5'ggg ttg acc taa ttc ccc agt atc3'
(SEQ ID NO: 8)

Primer M3R: 5'gat act ggg gaa gTa ggt cag acc c3' (SEQ ID NO: 9)

Oligos for pYal-CALB**Primer C:**5'-cga Tga gat ttc ctt cca ttt -3'
(SEQ ID NO: 10)**Primer D:**

5'-5'tct aga aag gtg gcg gcc gcc -3' (SEQ ID NO: 11)

Oligos for error-prone PCR**Primer E:**5'gaa got gga ttc cat cat cat c3'
(SEQ ID NO: 12)**Primer D:**

5'-5'tct aga aag gtg gcg gcc gcc -3' (SEQ ID NO: 13)

FIG. 15B

23/26

Subtilisin E Nucleotide and Amino Acid Sequence

10	20	30	40	50	60	70	80
atgtctgtgcaggctgccgganaaaagcagtaacgaaaagaatacattgttcggatttaacagacaatgagtggaatgag							
M S V Q A A C K S S T E K K Y I V G F K Q T M S A M S							
90	100	110	120	130	140	150	160
ttcggccaagaaaaggatgttattttctgaaaagggcggaaggttcaaaagcaatttaagtatgttaacggcgccgag							
S A K K K D V I S E K G G K V Q K Q F K Y V N A A A							
170	180	190	200	210	220	230	240
caacattggatgaaaagctgttaaaagaattgaaaaagatccgagcgttgcatatgttggaagaagatcatattgcacat							
A T L D E K A V K E L K K D P S V A Y V E E D H I A H							
250	260	270	280	290	300	310	320
gaatatggcgaatctgttccctatggcattttctcaaatataagcgccggctcttcactctcaaggctacacaggtctaa							
E Y A Q S V P Y G I S Q I K A P A L H S Q G Y T G S N							
330	340	350	360	370	380	390	400
cgtaaaagtagctgttatcgacagcggaattgactctctcactctgacttaaagctgagaggcgagcaagcttctgac							
V K V A V I D S G I D S S H P D L N V R G G A S F V							
410	420	430	440	450	460	470	480
cttctgaaacaaaccataccaggacggcaggtctctcaggtacgcatgttagccggtacgattgcgcgtcttataaactca							
P S E T N P Y Q D G S S H G T H V A G T I A A L N N S							
490	500	510	520	530	540	550	560
atcgggtgtttctggcggttagcccaagcgcatcattatgtcagtaaaagtgccttgattcaacagggaagcgcccaatatag							
I G V L G V S P S A S L Y A V K V L D E T G S G Q Y S							
570	580	590	600	610	620	630	640
ctggattattaaeggcatttgagtgggccatttccaaacaatatggatgttatcaacatgagccttggcggaactaactggtt							
W I I N G I E W A I S N N M D V I N M S L G G P T G							
650	660	670	680	690	700	710	720
ctacagcgctgaaaacagtcgttgacaaagccgtttccagcgggtatcgttcgttcgctgcgcagccggaaacgaaggttca							
S T A L K T V V D K A V S S G I V V A A A G N E G S							
730	740	750	760	770	780	790	800
tccggaagcaaaagacagtcggctaccctgcaaaatatccttctactattgcaagtaggtgcggttaaacagcagcaacca							
S G S T S T V G Y P A K Y P S T I A V G A V N S S N Q							
810	820	830	840	850	860	870	880
aagagcttcattctccagcgaggtttctgagcttgatgtgatggtctcctggcggtgcacatcaaaagcacacttctggag							
R A S F S S A G S E L D V M A P C V S I Q S T L P G							
890	900	910	920	930	940	950	960
gcacttaacggcgattataacggaacgtccatgggaactcctcagcttgccggagcagcagcgttaattcttttaagcac							
G T Y G A Y N G T S M A T P H V A G A A A L I L S K H							
970	980	990	1000	1010	1020	1030	1040
cegacttggaacaaacggcgcaagtcggtgatcgttttagaaagcactgcaacatatcttggaactctttctactatggaaa							
P T W T N A Q V R D R L E S T A T Y L G N S F Y Y G K							
1050	1060	1070					
aggggttaatacaacgtacaagcagctgaacaataa (SEQ ID NO: 14)							
G L I N V Q A A A Q * (SEQ ID NO: 15)							

FIG. 16A

24/26

Subtilisin Amino Acid Allgntment

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
ALA GLN SER VAL PRO TRP GLY ILE SER ARG VAL GLN ALA PRO ALA ALA HIS ASN
ALA GLN SER VAL PRO THR GLY ILE SER GLN ILE LYS ALA PRO ALA LEU HIS SER
ALA LYS CYS VAL SER THR GLY VAL SER GLN ILE LYS ALA PRO ALA LEU HIS SER

19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
ARG GLY LEU THR GLY SER GLY VAL LYS VAL ALA VAL LEU ASP THR GLY ILE SER
GLN GLY THR THR GLY SER ASN VAL LYS VAL ALA VAL ILE ASP SER GLY ILE ASP
GLN GLY THR THR GLY SER ASN VAL LYS VAL ALA VAL ILE ASP SER GLY ILE ASP

37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54
THR --- HIS PRO ASP LEU ASN ILE ARG GLY GLY ALA SER PHE VAL PRO GLY GLU
SER SER HIS PRO ASP LEU ASN VAL ARG GLY GLY ALA SER PHE VAL PRO SER GLU
SER SER HIS PRO ASP LEU ASN VAL ALA GLY GLY ALA SER PHE VAL PRO SER GLU

55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72
--- --- PRO SER THR GLN ASP GLY ASN GLY HIS GLY THR HIS VAL ALA GLY THR
THR ASN PRO THR --- GLN ASP GLY SER SER HIS GLY THR HIS VAL ALA GLY THR
THR ASN PRO PHE --- GLN ASP ASN ASN SER HIS GLY THR HIS VAL ALA GLY THR

73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90
ILE ALA ALA LEU ASN ASN SER ILE GLY VAL LEU GLY VAL ALA PRO ASN ALA GLU
ILE ALA ALA LEU ASN ASN SER ILE GLY VAL LEU GLY VAL SER PRO SER ALA SER
--- --- --- --- --- --- --- --- --- VAL LEU ALA VAL ALA PRO SER ALA SER

91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108
LEU THR ALA VAL LYS VAL LEU GLY ALA SER GLY SER GLY SER VAL SER SER ILE
LEU THR ALA VAL LYS VAL LEU ASP SER THR GLY SER GLY GLN THR SER TRP ILE
LEU THR ALA VAL LYS VAL LEU GLY ALA ASP GLY SER GLY GLN THR SER TRP ILE

109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126
ALA GLN GLY LEU GLU TRP ALA GLY ASN ASN GLY MET HIS VAL ALA ASN LEU SER
ILE ASN GLY ILE GLU TRP ALA ILE SER ASN ASN MET ASP VAL ILE ASN MET SER
ILE ASN GLY ILE GLU TRP ALA ILE ALA ASN ASN MET ASP VAL ILE ASN MET SER

127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144
LEU GLY SER PRO SER PRO SER ALA THR LEU GLU GLN ALA VAL ASN SER ALA THR
LEU GLY GLY PRO THR GLY SER THR ALA LEU LYS THR VAL VAL ASP LYS ALA VAL
LEU GLY GLY PRO SER GLY SER ALA ALA LEU LYS ALA ALA VAL ASP LYS ALA VAL

145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162
SER ARG GLY VAL LEU VAL VAL ALA ALA SER GLY ASN SER GLY --- ALA GLY SER (SEQ ID NO: 16)
SER SER GLY ILE VAL VAL ALA ALA ALA ALA GLY ASN GLU GLY SER SER GLY SER (SEQ ID NO: 17)
ALA SER GLY VAL VAL VAL VAL ALA ALA ALA GLY ASN GLU GLY THR SER GLY SER (SEQ ID NO: 18)

FIG. 16B

25/26

Subtilisin Amino Acid Alignment (cont'd.)

163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180
 ILE SER --- --- --- TYR PRO ALA ARG TYR ALA ASN ALA MET ALA VAL GLY ALA
 THR SER THR VAL GLY TYR PRO ALA LYS TYR PRO SER THR ILE ALA VAL GLY ALA
 SER SER THR VAL GLY TYR PRO GLY LYS TYR PRO SER VAL ILE ALA VAL GLY ALA

181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198
 THR ASP GLN ASN ASN ASN ARG ALA SER PHE SER GLN TYR GLY ALA GLY LEU ASP
 VAL ASN SER SER ASN GLN ARG ALA SER PHE SER SER ALA GLY SER GLU LEU ASP
 VAL ASP SER SER ASN GLN ARG ALA SER PHE SER SER VAL GLY PRO GLU LEU ASP

199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216
 ILE VAL ALA PRO GLY VAL ASN VAL GLN SER THR TYR PRO GLY SER THR TYR ALA
 VAL MET ALA PRO GLY VAL SER ILE GLN SER THR LEU PRO GLY GLY THR TYR GLY
 VAL MET ALA PRO GLY VAL SER ILE CYS SER THR LEU PRO GLY ASN LYS TYR GLY

217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234
 SER LEU ASN GLY THR SER MET ALA THR PRO HIS VAL ALA GLY ALA ALA ALA LEU
 ALA TYR ASN GLY THR CYS MET ALA THR PRO HIS VAL ALA GLY ALA ALA ALA LEU
 ALA LYS SER GLY THR SER MET ALA SER PRO HIS VAL ALA GLY ALA ALA ALA LEU

235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252
 VAL LYS GLN LYS ASN PRO SER TRP SER ASN VAL GLN ILE ARG ASN HIS LEU LYS
 ILE LEU SER LYS HIS PRO THR TRP THR ASN ALA GLN VAL ARG ASP ARG LEU GLU
 ILE LEU SER LYS HIS PRO ASN TRP THR ASN THR GLN VAL ARG SER SER LEU GLU

253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270
 ASN THR ALA THR SER LEU GLY SER THR ASN LEU TYR GLY SER GLY LEU VAL ASN
 SER THR ALA THR TYR LEU GLY ASN SER PHE TYR TYR GLY LYS GLY LEU ILE ASN
 ASN THR THR THR LYS LEU GLY ASN SER PHE TYR TYR GLY LYS GLY LEU ILE ASN

271 272 273 274 275 276
 ALA GLU ALA ALA THR ARG (SEQ ID NO: 16)
 VAL GLN ALA ALA ALA GLN (SEQ ID NO: 17)
 VAL GLN ALA ALA ALA GLN (SEQ ID NO: 18)

FIG. 16C

26/26

PCR Oligos for Subtilisin E

A primer-

5'-cag agc gttg cat atg tgg aag-3'
(SEQ ID NO: 19)

B-primer-

5'-tta gga tcc tta atg atg atg atg atg atg atg atg
agc tgc tgc tgc gtt gat-3' (SEQ ID NO: 20)

1- K27Y

F 5'-ggc tct aac gta TAT gta gct gtt atc-3'
(SEQ ID NO: 21)
R 5'-gat aac agc tac ATA tac gtt aga gcc-3'
(SEQ ID NO: 22)

5.1- G61Y

F 5'-cca tac cag gaa TAc agt tct cac gg-3' (SEQ ID NO: 33)
R 5'-ca gtc aga act gTA gtc ctg gta tgg-3' (SEQ ID NO: 34)

2- K237Y

F 5'-tta att ctt tct TcC cac cgc act tgg-3'
(SEQ ID NO: 23)
R 5'-cca agt cgg gtc GtA aga aag aat taa c-3'
(SEQ ID NO: 24)

5.2- S98Y

F 5'-aa gtc ctt gat TAT aca gga agc ggc-3' (SEQ ID NO: 35)
R 5'-gcc gct tcc tgc ATA aac nag cac tt-3' (SEQ ID NO: 36)

3.1- D36Y

F 5'-gac ago gga att T act ctt ctc atc-3'
(SEQ ID NO: 25)
R 5'-gat gag aag agt A aat tcc gct gtc-3'
(SEQ ID NO: 26)

6.1- H17Y

F 5'-gcg cgc gct ctt Tac tct cca ggc t-3' (SEQ ID NO: 37)
R 5'-a gcc tgc aga gTA nag agc cgg cgc-3' (SEQ ID NO: 38)

3.2- P210Y

F 5'-caa agc aca ctt TAt gga ggc act tac-3'
(SEQ ID NO: 27)
R 5'-ta agt gcc tcc aTA aag tgc gct tgc-3'
(SEQ ID NO: 28)

6.2- P86Y

F 5'-ctg ggc gtt agc TAT ago gca tca tta-3' (SEQ ID NO: 39)
R 3'-taa tga tgc gct ATA gct aac gcc cag-3' (SEQ ID NO: 40)

4.1- K170Y

F 5'-ggc tac cct gca TAT tat cct tct act a-3'
(SEQ ID NO: 29)
R 5'-agt aga agc ata ATA tgc agg gta gcc-3'
(SEQ ID NO: 30)

7- P201Y

F 5'-gat gtc atg gct TAt ggc gtc tcc atc-3' (SEQ ID NO: 41)
R 5'-gat gga cac gcc aTA agc cat cac atc-3' (SEQ ID NO: 42)

4.2- E195Y

F 5'-agc gca ggt tct TAT ctt gat gtc atg-3'
(SEQ ID NO: 31)
R 5'-cat cac atc aag ATA aga acc tgc gct-3'
(SEQ ID NO: 32)

FIG. 16D